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Oxidation of uroporphyrinogen by methylcholanthrene-induced cytochrome *P*-450

Essential role of cytochrome *P*-450*d*

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We have previously shown that uroporphyrinogen is oxidized to uroporphyrin by microsomes (microsomal fractions) from 3-methylcholanthrene-pretreated chick embryo liver [Sinclair, Lambrecht & Sinclair (1987) Biochem. Biophys. Res. Commun. **146**, 1324–1329]. We report here that a specific antibody to chick liver methylcholanthrene-induced cytochrome *P*-450 (*P*-450) inhibited both uroporphyrinogen oxidation and ethoxyresorufin *O*-de-ethylation in chick-embryo liver microsomes. 3-Methylcholanthrene-pretreatment of rats and mice markedly increased uroporphyrinogen oxidation in hepatic microsomes as well as *P*-450-mediated ethoxyresorufin de-ethylation. In rodent microsomes, uroporphyrinogen oxidation required the addition of NADPH, whereas chick liver microsomes required both NADPH and 3,3′4,4′-tetrachlorobiphenyl. Treatment of rats with methylcholanthrene, hexachlorobenzene and *o*-aminoazotoluene increased uroporphyrinogen oxidation and *P*-450*d*, whereas phenobarbital did not increase either. The contribution of hepatic *P*-450*c* and *P*-450*d* to uroporphyrinogen oxidation and ethoxyresorufin *O*-de-ethylation in methylcholanthrene-induced microsomes was assessed by using specific antibodies to *P*-450*c* and *P*-450*d*. Uroporphyrinogen oxidation by methylcholanthrene-induced rat liver microsomes was inhibited up to 75% by specific antibodies to *P*-450*d*, but not by specific anitbodies to *P*-450*c*. In contrast, ethoxyresorufin de-ethylation was inhibited only 20% by anti-*P*450*d* but 70% by anti-*P*450*c*. Methylcholanthrene-induced kidney microsomes which contain *P*-450*c* but non *P*-450*d* did not oxidize uroporphyrinogen. These data indicate that hepatic *P*-450*d* catalyses uroporphyrinogen oxidation. We suggest that the *P*-450*d*-catalysed oxidation of uroporphyrinogen has a role in the uroporphyria caused by hexachlorobenzene and other compounds.

INTRODUCTION

One manifestation of exposure of humans and experimental animals to certain polyhalogenated aromatic compounds (PHAs) is a porphyria which is characterized by hepatic accumulation and urinary overexcretion of uroporphyrin and heptacarboxyporphyrin (for a review, see Sweeney, 1986). A role for cytochrome *P*-450 (*P*-450) in PHA-mediated uroporphyria was suggested from experiments with chick-embryo liver-cell cultures (Sinclair & Granick, 1974). Inhibitors of *P*-450 prevented the accumulation of uroporphyrin caused by PHAs (Sinclair & Granick, 1974; Debets *et al.*, 1980). The form of *P*-450 induced by 3-methylcholanthrene (MC) in chick-embryo liver cultures was implicated in the accumulation of uroporphyrin (Sinclair *et al.*, 1984). Moreover, in rodents, susceptibility to PHA-induced porphyria is inherited in parallel with inducibility of aryl-hydrocarbon hydroxylase, an activity catalysed by PHA-induced *P*-450 (Jones & Sweeney, 1980).

The mechanism by which induced *P*-450 causes uroporphyria is not known, but presumably involves some disturbance in the metabolism of the haem-biosynthetic intermediate, uroporphyrinogen. The activity of the enzyme uroporphyrinogen decarboxylase, which converts uroporphyrinogen into coproporphyrinogen, is decreased in rodents made uroporphyric by treatment with PHAs (Elder *et al.*, 1976; Sweeney & Jones, 1977). This decrease in catalytic activity of uroporphyrinogen decarboxylase does not appear to be accompanied by a decrease in enzyme protein as determined immunochemically (Elder & Shepherd, 1982). Stable inhibitors of uroporphyrinogen decarboxylase have been identified in the livers of PHA-treated porphyric rodents (Cantoni *et al.*, 1984; Billi *et al.*, 1986; Smith & Francis, 1987) and it has been suggested that such inhibitors may be the result of *P*-450-mediated metabolism of PHAs (Cantoni *et al.*, 1984; Billi *et al.*, 1986). However, Francis & Smith (1987) recently proposed that uroporphyrinogen decarboxylase is inhibited by a porphyrin derivative formed as the result

Abbreviations used: DMSO, dimethyl sulphoxide; EROD, 7-ethoxyresorufin de-ethylase; HCBZ, hexachlorobenzene; MC, 3-methylcholanthrene; β-NF, β-naphthoflavone; OAT, *o*-aminoazotoluene; PB, phenobarbital; PHA, polyhalogenated aromatic hydrocarbon; TCB, 3,3′,4,4′-tetrachlorobiphenyl; *P*-450, cytochrome *P*-450; i.p., intraperitoneal.

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of a chronic oxidative process due to uncoupling of *P*-450-mediated electron transport.

By contrast, in birds, we have recently found conditions where the development of uroporphyria is not accompanied by uroporphyrinogen decarboxylase inhibition (Lambrecht *et al.*, 1988). After treatment with PHAs, high levels of uroporphyrin accumulate in chick-embryo liver cultures and Japanese-quail liver without a decrease in the activity of uroporphyrinogen decarboxylase. This finding suggested that the primary cause of uroporphyria in these PHA-treated avian systems is not the inhibition of uroporphyrinogen decarboxylase. We have found that liver microsomes (microsomal fractions) from chick embryos treated with MC catalysed the oxidation of uroporphyrinogen to uroporphyrin in the presence of NADPH and TCB (Sinclair *et al.*, 1987). By contrast, neither microsomes from untreated embryos nor microsomes from embryos treated with a phenobarbital-like inducer oxidized uroporphyrinogen. These results have been confirmed by De Matteis *et al.* (1988), who also reported that microsomes from β-naphthoflavone (β-NF)-pretreated rats oxidized uroporphyrinogen to uroporphyrin in the presence of NADPH. However, since microsomes from control rats were not tested, it was not possible to conclude whether the β-NF pretreatment was necessary.

The present studies were designed to determine whether the ability of rodent liver microsomes to catalyse uroporphyrinogen oxidation required PHA pretreatment of the animal and to determine whether one of the PHA-induced isoenzymes of *P*-450 was responsible for uroporphyrinogen oxidation. We report here that microsomes isolated from rats and mice pretreated with MC, hexachlorobenzene (HCBZ) or *o*-aminoazotoluene (OAT) oxidized uroporphyrinogen to uroporphyrin. These compounds induce two highly homologous isoenzymes of *P*-450 in rats, namely *P*-450*c* and *P*-450*d* (Thomas *et al.*, 1983; Linko *et al.*, 1986; Degawa *et al.*, 1987). Antibody inhibition studies indicated that only the *P*-450*d* isoenzyme was responsible for increased oxidation of uroporphyrinogen.

MATERIAL AND METHODS

Chemicals

3,3′4,4′-Tetrachlorobiphenyl was purchased from Ultra-Science (Hope, RI, U.S.A.). Ethoxyresorufin was purchased from Molecular Probe (Eugene, OR, U.S.A.) Uroporphyrin I was purchased from Porphyrin Products (Logan, UT, U.S.A.). Dimethyl sulphoxide (DMSO) (Puriss grade) was purchased from Fluka (Hauppauge, NY, U.S.A.). Hexachlorobenzene (HCBZ; organic analytic standard grade) was purchased from BDH (Poole, Dorset, U.K.). *o*-Aminoazotoluene (OAT) was purchased from Aldrich (Milwaukee, WI, U.S.A.). Glutethimide, phenobarbital (PB), 3-methylcholanthrene (MC), isocitrate dehydrogenase, sodium isocitrate, nicotinamide and NADPH (Type III) were purchased from Sigma (St. Louis, MO, U.S.A.). Reagents for electrophoresis were purchased from Bio-Rad (Richmond, CA, U.S.A.).

Animals, treatments, and preparation of microsomes

Fisher 344 female rats (100–150 g) were obtained from Charles River Breeding Laboratories (Willmington, MA, U.S.A.). MC was dissolved in warm corn oil (12 mg/ml) and given as a single intraperitoneal (i.p.) dose (130 mg/kg) 48 h before the animals were killed by decapitation (Urquhart *et al.*, 1988). HCBZ, dissolved in warm corn oil (12 mg/ml) was given as a single i.p. dose (350 mg/kg) 72 h before the rats were killed (Li *et al.*, 1986). OAT (5 mg/ml in corn oil) was given as a single i.p. dose (150 mg/kg) 24 h before the animals were killed (Kelley *et al.*, 1987). Sodium phenobarbital (PB) (10 mg/ml of 0.9% NaCl) was administered i.p. at 100 mg/kg for 3 consecutive days, with the rats being killed 24 h after the third treatment (Urquhart & Elder, 1987). C57BL/6 male mice obtained from Charles River Breeding Laboratories were treated with MC or corn oil as described above. White-Leghorn-chick embryos, 15–16 days old, were injected with 0.5 mg of MC in DMSO or DMSO alone (0.2 ml) 48 h before the animals were killed. Livers were homogenized in 0.25 M-sucrose/0.05 M-Hepes/1 mM-EDTA, pH 7.6, and microsomes were prepared by differential centrifugation as previously described (Sinclair *et al.*, 1979). Microsomes were stored at −70 °C as suspensions in 20% (v/v) glycerol/0.1 M-sodium phosphate/0.1 mM-EDTA, pH 7.4, until used, usually within 1 week.

Preparation of antibodies

P-450*d* was purified from 3,3′,4,4′,5,5′-hexachlorobiphenyl-treated rats and *P*-450*c* was purified from MC-treated rats (Goldstein *et al.*, 1982). Antiserum to *P*-450*d* was raised in goats and to *P*-450*c* in rabbits. IgG was prepared as described by Thomas *et al.* (1979). Since *P*-450*c* and *P*-450*d* are immunologically related (Luster *et al.*, 1983), specific antibody to *P*-450*d* (anti-*P*-450*d*) was obtained by repeated immunoadsorption over solid-phase immunoaffinity columns prepared from purified *P*-450*c* coupled to CNBr-activated Sepharose 4B (Luster *et al.*, 1983) and specific antibody to *P*-450*c* (anti-*P*-450*c*) was prepared by immunoadsorption with *P*-450*d* coupled to CNBr-activated Sepharose. Immunopurified anti-*P*-450*d* contained < 3% cross reactivity to *P*-450*c* as verified by a dot-blot procedure with slight modifications (Domin *et al*, 1984) and immunoblots by the method of Towbin *et al.* (1979). Cross-reactivity of anti-*P*-450*c* with *P*-450*d* was < 1%. The MC-induced *P*-450 from MC-treated chick-embryo liver was isolated as described in the preceding paper (Sinclair *et al.*, 1989) and antibodies against it were raised in rabbits. IgG was prepared from serum by affinity chromatography on protein A (Ey *et al.*, 1978).

Immunoblotting

Discontinuous SDS/polyacrylamide-gel electrophoresis was performed in a Bio-Rad Minigel apparatus, using a 10% (w/v) acrylamide separating gel. After electrophoresis at 200 V for 1 h, proteins were transferred to nitrocellulose sheets at 100 V for 1 h (Towbin *et al.*, 1979). The nitrocellulose sheets containing the transferred microsomal proteins were rinsed in 0.02 M-Tris/0.5 M-NaCl buffer, pH 7.4 (TBS) for 5 min, blocked in 5% powdered non-fat milk in TBS containing 0.5% Tween 20 for 1 h at room temperature, rinsed in TBS for 5 min, and then incubated with IgG. After 1 h at room temperature, the sheets were washed three times with TBS, and incubated with alkaline phosphatase-linked IgG. The blots were revealed using the Bio-Rad alkaline phosphatase Immune-Blot assay system.

Assays

Oxidation of uroporphyrinogen to uroporphyrin was monitored at room temperature in a Perkin–Elmer 650–10S spectrofluorimeter (excitation 397 nm, emission 417 nm) with the excitation slit at 2 nm and the emission slit at 15 nm. Uroporphyrin I was used as a standard. The assay mixture contained 1 ml of assay buffer (0.25 M-sucrose/0.05 M-Hepes/1 mM-EDTA, pH 7.6), 0.01 ml of microsomal suspension containing 0.15–0.2 mg of protein, 0.02 ml of NADPH-generating system [125 mg of sodium isocitrate, 40 mg of nicotinamide, 35 mg of NADPH, 70 mg of $MgCl_2$,$7H_2O$ and 0.2 ml of isocitrate dehydrogenase (20 units) dissolved in 0.75 ml of assay buffer]. Uroporphyrinogen I was made from uroporphyrin I by reduction with sodium amalgam (Sinclair *et al.*, 1987). With rodent microsomes, the reaction was initiated by addition of uroporphyrinogen to give a final concentration of 2 μM, and the increase in uroporphyrin fluorescence was measured for up to 30 min. With chick microsomes the reaction was started by the addition of 0.01 ml of 3,3′4,4′-tetrachlorobiphenyl (TCB) (0.2 mg/ml solution in DMSO). Ethoxyresorufin de-ethylase (EROD) activity was measured spectrofluorimetrically (excitation 530 nm, emission 587 nm, slits 10 nm) using a modification of the assay procedure of Burke *et al.* (1985). The assay mixture was the same as in the uorporphyrinogen oxidation assay, except for the addition of 1 mg of bovine serum albumin/ml (final concn.). The reaction was started by the addition of 3 μl of 1 mM-ethoxyresorufin in DMSO to give a final concentration of 3 μM, and the fluorescence was measured for 2–5 min. Resorufin was used as a standard. Antibody inhibition of catalytic activity was determined by incubating IgG and microsomal suspensions in a minimal volume (90–120 μl) of assay buffer containing preimmune IgG and specific IgG for 10 min at room temperature, after which the other reagents and buffer were added to a final volume of 0.25 ml. The total amount of IgG was kept constant. All assays were performed within the linear ranges of incubation time and protein concentration.

Protein concentrations were determined by the method of Lowry *et al.* (1951), with bovine serum albumin as a standard. *P*-450 concentrations were determined from CO-minus-reduced difference spectra as described by Omura & Sato (1964). *P*-450*c* and *P*-450*d* contents were assayed by radioimmunoassay as described previously (Luster *et al.*, 1983). The data were analysed by using Student's *t* test.

RESULTS AND DISCUSSION

Inhibition of uroporphyrinogen oxidation in chick-embryo liver microsomes by antibody specific for the MC-induced form of *P*-450

Previous work has suggested that uroporphyrinogen oxidation by chick-embryo liver microsomes is dependent on the presence of the MC-induced form of *P*-450 (Sinclair *et al.*, 1987). Fig. 1 indicates that both uroporphyrinogen oxidation and EROD were completely inhibited by antibody to the MC-induced *P*-450 of chick-embryo liver. Fig. 2 demonstrates that the antibody used in these studies was specific for the MC-induced form of embryonic chick liver *P*-450. Only a single band, which was extremely faint in both uninduced microsomes and microsomes from embryos treated with glutethimide, was detected in MC-induced microsomes. The observations that both activities catalysed by chick microsomes were completely inhibited by this antibody and that this antibody recognized only one protein in MC-treated chick microsomes suggest that a single *P*-450 induced by MC is responsible for both activities.

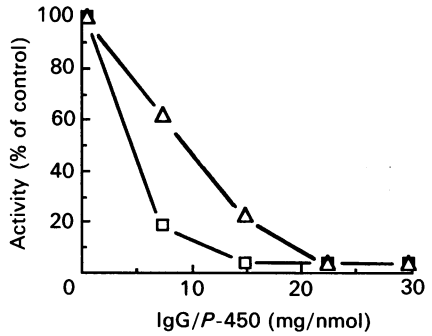

Fig. 1. Immunotitration of chick-embryo liver uroporphyrinogen oxidation and EROD

Liver microsomes from MC-treated chick embryos were preincubated with various amounts of IgG raised against chick MC-induced *P*-450 for 10 min at room temperature. Uroporphyrinogen oxidation (△) and EROD (□) were assayed as described in the Materials and Methods section; 100% EROD activity was 1695 pmol/min/mg of protein; 100% uroporphyrinogen oxidation activity was 36 pmol/min per mg of protein. The total amount of IgG (preimmune plus immune) was constant in all incubations.

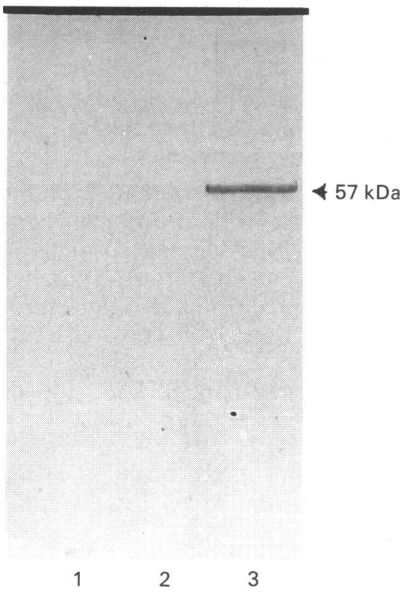

Fig. 2. Immunoblot of microsomal *P*-450 isoenzymes induced in chick-embryo liver

The amount of protein was 10 μg/lane for each microsomal sample. IgG against chick MC-induced *P*-450 was used. Lane 1 contained control microsomes; lane 2, microsomes from glutethimide-treated chick embryos; and lane 3, microsomes from MC-treated chick embryos. The immunoblotting procedure is described in the Materials and methods section.

Table 1. Comparison of uroporphyrinogen oxidation and EROD by liver microsomes from methylcholanthrene-treated rats, mice and chick embryos

Animals were treated as described in the Materials and methods section. The values reported represent the means ± S.D. of activities of hepatic microsomes from three rats, four mice and two groups of six chick-embryos per treatment. Assays of uroporphyrinogen oxidation and EROD activity are described in the Materials and methods section; 0.15–0.2 mg of microsomal protein/ml was used in all assays.

		Activity (pmol/min per mg of protein)	
Species	Treatment	Uroporphyrinogen oxidation	EROD
Rat	Corn oil	2.0 ± 0.7	137 ± 60
	MC	25.0 ± 8.0	2342 ± 600
Mouse	Corn oil	13.4 ± 3.2	224 ± 128
	MC	50.7 ± 11.4	4265 ± 1726
Chick embryo	DMSO	1.8 ± 0.6*	0
	MC	46.0 ± 9.0*	1895 ± 160

* In the presence of TCB (2 μg/ml).

Effect of MC treatment on uroporphyrinogen oxidation and ethoxyresorufin de-ethylation by rat, mouse and chick-embryo liver microsomes

Table 1 shows that pretreatment of animals with MC caused an increase in both uroporphyrinogen oxidation and EROD activity in liver microsomes of rats, mice and chick embryos. In a separate experiment microsomes from untreated mice had the same uroporphyrinogen oxidation rate as those from corn-oil-treated mice (results not shown). EROD was used as a marker for the induction of *P*-450 by PHAs. In all experiments, uroporphyrinogen oxidation was dependent on the addition of NADPH (results not shown). In rat liver microsomes, for approximately the first 5 min, there was a low, non-linear, rate of oxidation, which was followed by a linear higher rate, in agreement with the findings of De Matteis *et al.* (1988). This latter higher rate is shown in the Tables. Uroporphyrinogen oxidation by chick-embryo liver differed from that of rodents in that TCB, as well as NADPH, was required for the oxidation to proceed, as noted previously (Sinclair *et al.*, 1987; De Matteis *et al.*, 1988). TCB was added in DMSO and there was a low oxidation rate with DMSO alone (< 2 pmol/min per mg of protein). In rodents, TCB or DMSO alone was slightly inhibitory.

Effect of OAT, HCBZ and PB on uroporphyrinogen oxidation and EROD activity in rat liver microsomes

To investigate whether a particular isoenzyme of cytochrome *P*-450 was involved in uroporphyrinogen oxidation by rat liver microsomes, we treated rats with agents known to be preferential inducers of different isoenzymes of cytochrome *P*-450. In the rat, MC, HCBZ, and OAT induce isoenzymes of the *P*-450 I family (Thomas *et al.*, 1983; Linko *et al.*, 1986; Degawa *et al.*, 1986). Phenobarbital (PB) and HCBZ induce isoenzymes from the *P*-450 II family (Nebert *et al.*, 1987). MC, HCBZ, and OAT have been found to induce different proportions of two isoenzymes of the IA subfamily. These isoenzymes (IA_1 and IA_2) are also known as *P*-450*c* and *P*-450*d* in rat, and cytochromes $P\text{-}450P_1$ and $P\text{-}450P_3$ in mouse (Thomas *et al.*, 1983; Kimura *et al.*, 1984; Degawa *et al.*, 1986; Linko *et al.*, 1986). In the rat, MC induces both *P*-450*c* and *P*-450*d*, but the hepatic content of *P*-450*c* is somewhat greater than that of *P*-450*d* (Thomas *et al.*, 1983; Luster *et al.*, 1983). In comparison, OAT and HCBZ are better inducers of *P*-450*d* than *P*-450*c* (Luster *et al.*, 1983; Degawa *et al.*, 1986; Linko *et al.*, 1986; Kelley *et al.*, 1987).

Table 2 shows that microsomes from rats treated with MC, HCBZ, and OAT oxidized uroporphyrinogen at rates 5–8-fold higher than did corn-oil-treated controls. By contrast, PB did not induce this activity. These results suggest that only isoenzymes of the *P*-450 I family catalyse uroporphyrinogen oxidation. The increase in uroporphyrinogen oxidation is remarkably similar to the increase in *P*-450*d*. By contrast, MC produces a much larger increase in *P*-450*c* and EROD than did either HCBZ or OAT. These data are consistent with previous reports that *P*-450*c* has a 10-fold higher EROD activity per mol than cytochrome *P*-450*d* (Goldstein *et al.*, 1982). The relative magnitude of the increases in liver microsomal uroporphyrinogen oxidation, EROD activity,

Table 2. Effect of inducers of cytochromes *P*-450 on uroporphyrinogen oxidation and EROD activities of rat liver microsomes

The treatments and assays are described in the Materials and methods section. The values reported represent the means ± S.D. of microsomal activities of three animals per treatment. Three uroporphyrinogen oxidation assays and two EROD assays of microsomes were performed on each animal; 0.15–0.2 mg of microsomal protein/ml was used. The amounts of *P*-450*c* and *P*-450*d* were determined by radioimmunoassay. **P* < 0.01 (significant increase when compared with corn oil-treated animals).

	Activity (pmol/min per mg of protein)		Cytochrome (nmol/mg of protein)		
Treatment	Uroporphyrinogen oxidation	EROD	Total *P*-450	*P*-450*d*	*P*-450*c*
Corn oil	2.8 ± 1.0	70 ± 43	0.65 ± 0.05	0.038 ± 0.003	0.003 ± 0.001
MC	23.6 ± 7.0*	2176 ± 641*	0.90 ± 0.08*	0.270 ± 0.02*	0.470 ± 0.07*
PB	4.6 ± 1.6	92 ± 32	1.41 ± 0.23	0.022 ± 0.001	< 0.001
HCBZ	14.1 ± 4.1*	226 ± 30*	0.68 ± 0.08	0.141 ± 0.004*	0.030 ± 0.004*
OAT	18.9 ± 3.3*	173 ± 52*	0.87 ± 0.10*	0.270 ± 0.03*	0.060 ± 0.004*

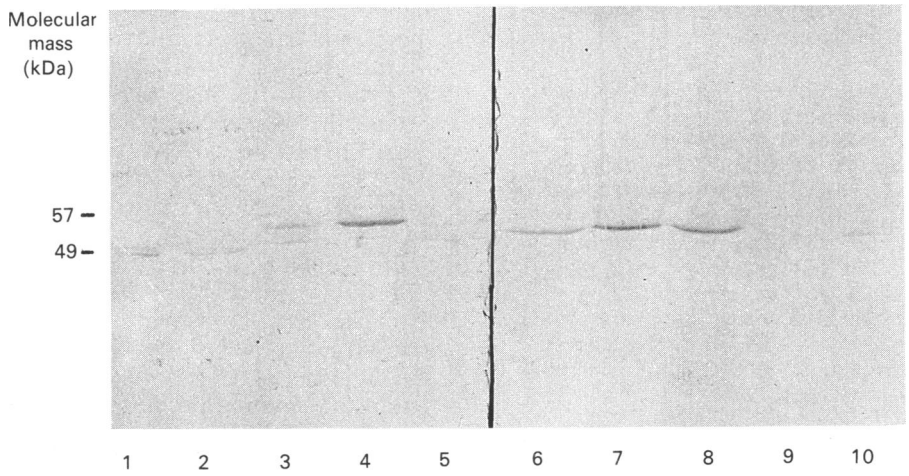

Fig. 3. Immunoblot of microsomal *P*-450 isoenzymes induced in rat liver

The amount of microsomal protein was 10 μg/well. One half of the nitrocellulose sheet was treated with monospecific anti-*P*-450*c*, and the other, with monospecific anti-*P*-450*d*. Lanes 1 and 10 contained control microsomes. Lanes 2 and 9 contained microsomes from PB-treated rats; lanes 3 and 8 contained microsomes from OAT-treated rats; lanes 4 and 7 contained microsomes from MC-treated rats; and lanes 5 and 6 contained microsomes from HCBZ-treated rats. Lanes 1–5 were treated with anti-*P*-450*c*; lanes 6–10 were treated with anti-*P*-450*d*. The M_r of cytochrome *P*-450*c* is 56000; of cytochrome *P*-450*d*, 52000.

and cytochromes *P*-450*c* and *P*-450*d* suggests that increases in *P*-450*d* might account for the increases in uroporphyrinogen oxidation.

Effect of antibodies specific for *P*-450 isoenzymes *c* and *d* on uroporphyrinogen oxidation

To determine more conclusively which isoenzyme of cytochrome *P*-450 was involved in uroporphyrinogen oxidation, increasing concentrations of specific antibodies against *P*-450*c* and *P*-450*d* were used to inhibit uroporphyrinogen oxidation and EROD activity in hepatic microsomes from MC-treated rats. Neither anti-*P*-450*c* nor anti-*P*-450*d* recognized the heterologous cytochrome on immunoblots (Fig. 3). Dot-blots revealed < 1% cross-reactivity of anti-P-450*c* with P-450*d* and < 3% cross-reactivity of anti-P450*d* with P-450*c* (results not shown).

Uroporphyrinogen oxidation was inhibited by at least 75% by anti-P-450*d*, whereas EROD activity was inhibited by only 20% (Fig. 4*a*), in agreement with the expected contribution of isoenzyme *d* to EROD activity in MC-induced rat liver microsomes (Thomas *et al.*, 1983; Kelley *et al.*, 1987). By contrast, anti-*P*-450*c* did not inhibit uroporphyrinogen oxidation, but inhibited EROD activity by at least 70% (Fig. 4). From these results we conclude that uroporphyrinogen oxidation induced by MC treatment in rat liver microsomes is catalysed by *P*-450*d*, but is not catalysed by P-450*c*.

The antibody to P-450*c* did cross-react with an additional protein of lower molecular mass in microsomes from control and treated animals. This protein probably represents a constitutive *P*-450 but its molecular mass differs from that of *P*-450*d* and it was not detected by anti-*P*-450*d*.

Uroporphyrinogen oxidation by kidney microsomes induced for *P*-450*c*

To provide additional evidence that *P*-450*c* does not

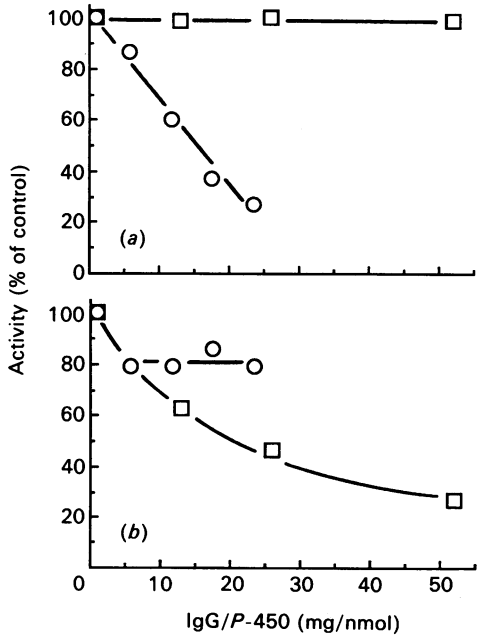

Fig. 4. Immunotitration of rat liver uroporphyrinogen oxidation and EROD

Liver microsomes from MC-treated rats were preincubated with various amounts of IgG raised against cytochrome *P*-450*c* (□) and *P*-450*d* (○) for 10 min at room temperature. Then uroporphyrinogen oxidation (*a*) and EROD (*b*) were assayed as described in the Materials and methods section. The means and ranges of 100% activities were 1527 (1398, 1656) pmol/min per mg of protein for EROD, and 30 (29, 32) pmol/min per mg of protein for uroporphyrinogen oxidation. The total amount of IgG (pre-immune plus immune) was constant in all incubations.

Table 3. Comparison of uroporphyrinogen oxidation and EROD in rat kidney and liver microsomes

EROD activity of MC-induced rat liver and kidney microsomes were made equal per unit volume by adding differing amounts of microsomes to the 1 ml volume. For measurement of control EROD, the amount of protein per assay was 0.26 mg/ml; for EROD of MC-treated liver microsomes, the amount of protein per assay was 0.125 mg/ml; for MC-treated kidney microsomes, the amount of protein per assay was 1.38 mg/ml. These concentrations of protein were also used to measure uroporphyrinogen oxidation in microsomes from both organs using the assay procedure described in the Materials and methods section. Values represent the means and ranges of duplicate determinations.

Organ and treatment	Activity (pmol/min per ml)	
	Uroporphyrinogen oxidation	EROD
Liver microsomes		
Control	0.6 (0.5, 0.7)	18 (18, 18)
MC	4.3 (4.3, 4.3)	356 (326, 385)
Kidney microsomes		
Control	< 0.5*	18 (18, 18)
MC	< 0.5*	296 (289, 303)

* Limit of detection.

catalyse uroporphyrinogen oxidation, we exploited the fact that, in rats, *P*-450*c*, but not *P*-450*d*, is induced by PHAs in extrahepatic tissue such as the kidney (Goldstein & Linko, 1984; Degawa *et al.*, 1987). Table 3 shows that MC increased both uroporphyrinogen oxidation and EROD activity in rat liver microsomes. However, kidney microsomes from the same rat exhibited an increase in EROD activity, but no increase in uroporphyrinogen oxidation. A mixture of MC-induced microsomes from both organs oxidized uroporphyrinogen at the same rate as the liver microsomes alone (results not shown). These data are consistent with the antibody-inhibition studies and provide further evidence that *P*-450*c* does not oxidize uroporphyrinogen.

General discussion

A number of earlier studies suggested that there is some correlation between the induction of *P*-450 by PHAs and the development of porphyria (Sweeney & Jones, 1977; Greig *et al.*, 1984; Smith & Francis, 1987). However, these studies assumed that P_1-450 (or P-450*c*) was involved. In comparisons of inbred mouse strains, inducibility of neither EROD activity, nor of aryl-hydrocarbon hydroxylase, an activity catalysed primarily by P_1-450, completely correlated with the porphyria caused by HCBZ or 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (Greig *et al.*, 1984; Smith & Francis, 1987). Both EROD activity and aryl-hydrocarbon hydroxylase activity in rats are catalysed mainly by the *c* isoenzyme (Thomas *et al.*, 1983). Our results point to an association of the uroporphyria with rat P-450*d* rather than with *P*-450*c*. Recent studies of the porphyric action of HCBZ in congenic strains of C57BL/6J mice, differing only at the *Ah* locus, also suggested that the porphyria induced by HCBZ may correlate more closely with the relative induction of P_3-450 than P_1-450 (Hahn *et al.*, 1988).

Since we have found conditions where PHA-induced uroporphyria can occur in avians without a decrease in uroporphyrinogen decarboxylase (Lambrecht *et al.*, 1988), we propose that uroporphyrinogen oxidation could be the primary cause of uroporphyria in these species. In rodents, PHAs cause a decrease in uroporphyrinogen decarboxylase activity. Recently it has been suggested that, during the oxidation of uroporphyrinogen, unstable, partially oxidized intermediates may form and bind to the active site of uroporphyrinogen decarboxylase, inactivating the enzyme (Urquhart *et al.*, 1988). The heat-stable inhibitor of uroporphyrinogen decarboxylase reported by Cantoni *et al.* (1984) and Smith & Francis (1987) may be formed from porphyrinogen oxidation products. We propose that, in the rodent, it is possible that uroporphyrinogen oxidation is the initial cause of uroporphyria, with uroporphyrinogen decarboxylase inactivation being a secondary event.

The mechanism of uroporphyrinogen oxidation by cytochrome *P*-450 is unknown. It has been suggested that binding of certain substrates by cytochrome *P*-450 can uncouple electron transport and lead to formation of active oxygen species (Sousa & Marletta, 1985). This possibility suggests a role for oxygen radicals in rodent uroporphyrinogen oxidation, but preliminary experiments with chick-embryo liver microsomal extracts indicated that hydrogen peroxide, superoxide and hydroxyl radical were not involved (Sinclair *et al.*, 1987); however, it is possible that radical scavengers and inhibitors may not penetrate to the membrane site of uroporphyrinogen oxidation.

Recently, it has been reported that certain compounds which induce cytochrome P-450*d* also bind tightly to the enzyme and do not detach during purification (Voorman & Aust, 1987). The *P*-450*d* isoenzyme of rat and its analogue, LM_4, in rabbits, can be isolated as stable complexes with halogenated biphenyls or MC bound to the cytochrome (Voorman & Aust, 1987; Imai *et al.*, 1980). It has been proposed that the stability of the complex decreases metabolic breakdown of the *P*-450 and may be responsible for some of the increase in *P*-450*d* seen after administration of PHAs (Voorman & Aust, 1988). Such stable complexes, if present in the rodent microsomes used in our assays, might explain the fact that TCB is required to initiate uroporphyrinogen oxidation by chick-embryo liver microsomes, but not by rat microsomes. Further work will be required to determine whether such complexes are involved in uroporphyrinogen oxidation.

In conclusion, the present studies show MC treatment increased the oxidation of uroporphyrinogen to uroporphyrin by liver microsomes of rats and mice as well as chick embryos. Oxidation by rodent liver microsomes required addition of NADPH for this reaction, whereas the oxidation by chick-embryo liver microsomes required both NADPH and TCB. Treatment with HCBZ or OAT also increased uroporphyrinogen oxidation by rodent liver microsomes. In chick-embryo liver, MC appeared to induce only one form of *P*-450 which was responsible for both EROD activity and uroporphyrinogen oxidation. In rats, the oxidation of uroporphyrinogen in MC-induced microsomes can be inhibited by antibody to *P*-450*d* but not by antibody to *P*-450*c*. These results implicate P-450*d* in the oxidation of uroporphyrinogen by rat liver microsomes.

Note added in proof (Received 3 January 1989)

Recently Hokama *et al.* (1988) reported the induction of *c*- and *d*-like forms of cytochrome *P*-450 by 3,3′,4,4′,5-pentachlorobiphenyl in 15-day-old chicks. The role of each of these forms in uroporphyrinogen oxidation remains to be investigated.

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REFERENCES

Billi, S. C., Wainstok de Calmanovici, R. & San Martin de Viale, S. C. (1986) in Hexachlorobenzene: Proceedings of an International Symposium (Morris, C. R. & Cabral, J. R. P., eds.) (IARC Scientific Publications no. 77), pp. 487–491, IARC, Lyon, France

Burke, M. D., Thompson, S., Elcomb, C. R., Halpert, J., Haaparanta, T. & Mayer, R. T. (1985) Biochem. Pharmacol. **34**, 3337–3345

Cantoni, L., Dal Fiume, D., Rizzardini, M. & Ruggieri, R. (1984) Toxicol. Lett. **20**, 211–217

Debets, F., Hamers, W. & Strik, J. (1980) Int. J. Biochem. **12**, 1019–1025

Degawa, M., Kojoma, M., Misaki, Y., Sato, Y. & Hashimoto, Y. (1986) Biochem. Pharmacol. **35**, 3565–3570

Degawa, M., Yamada, H., Hishinuma, T., Masuko, T. & Hashimoto, Y. (1987) J. Biochem. (Tokyo) **101**, 1437–1445

De Matteis, F., Harvey, C., Reed, C. & Hempenious, R. (1988) Biochem. J. **250**, 13–21

Domin, B. A., Serabjit-Singh, C. J. & Philpot, R. M. (1984) Anal. Biochem. **136**, 390–396

Elder, G. & Shepherd, D. M. (1982) Biochem. Biophys. Res. Commun. **109**, 113

Elder, G. H., Evans, J. & Martlin, S. A. (1976) Clin. Sci. Med. **51**, 71–80

Ey, P. L., Prowse, S. J. & Jenkin, R. C. (1978) Immunochemistry **15**, 429–436

Francis, J. & Smith, A. (1987) Biochem. Biophys. Res. Commun. **146**, 13–20

Goldstein, J. A. & Linko, P. (1984) Mol. Pharmacol. **25**, 185–191

Goldstein, J. A., Linko, P., Luster, M. T. & Sundheimer, D. W. (1982) J. Biol. Chem. **257**, 2702–2707

Greig, J. B., Francis, J. E., Kay, S. J. E., Lovell, D. P. & Smith, A. G. (1984) Toxicol. Appl. Pharmacol. **74**, 17–25

Hahn, M. E., Gasiewicz, T. A., Linko, P. & Goldstein, J. A. (1988) Biochem. J. **254**, 245–254

Hokama, Y., Koga, N. & Yoshimura, H. (1988) J. Biochem. (Tokyo) **104**, 355–361

Imai, Y., Hoshimoto-Yatsudo, C., Satoke, H., Gerardin, A. & Sato, R. (1980) J. Biochem. (Tokyo) **88**, 489–503

Jones, K. G. & Sweeney, G. D. (1980) Toxicol. Appl. Pharmacol. **53**, 42–49

Kelley, M., Hantelle, P., Safe, S., Levin, W. & Thomas, P. E. (1987) Mol. Pharmacol. **32**, 206–211

Kimura, S., Gonzalez, F. J. & Nebert, D. W. (1984) J. Biol. Chem. **249**, 10705–10713

Lambrecht, R. W., Sinclair, P. R., Bement, W. J., Sinclair, J. F., Carpenter, H. M., Buhler, D. R., Urquhart, A. J. & Elder, G. H. (1988) Biochem J. **253**, 131–138

Li, S. M. A., Denomme, M. A., Safe, S., Dutton, D., Parkinson, A., Thomas, P. E., Ryan, D., Bandiera, S. D., Reik, L. M. & Levin, W. (1986) in Hexachlorobenzene: Proceedings of an International Symposium (Morris, C. R. & Cabral, J. R. P., eds.) (IARC Scientific Publications no. 77), pp. 567–573, IARC, Lyon, France

Linko, P., Yeowell, H. N., Gasiewicz, T. A. & Goldstein, J. (1986) J. Biochem. Toxicol. **1**, 95–107

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) J. Biol. Chem. **193**, 265–275

Luster, M. I., Lawson, L. D., Linko, P. & Goldstein, J. (1983) Mol. Pharmacol. **23**, 252–257

Nebert, D. W., Adesnik, M., Coon, M. J., Estabrook, R. W., Gonzalez, F. J., Guengerich, F. P., Gunsalus, I. C., Johnson, E. F., Kemper, B., Levin, W., Phillips, I. R., Sato, R. & Waterman, M. R. (1987) DNA **6**, 1–6

Omura, T. & Sato, R. (1964) J. Biol. Chem. **239**, 2370–2378

Sinclair, P. & Granick, S. (1974) Biochem. Biophys. Res. Commun. **61**, 124–133

Sinclair, J. F., Sinclair, P. R. & Bonkovsky, H. L. (1979) Biochem. Biophys. Res. Commun. **87**, 710–714

Sinclair, P., Bement, J., Bonkovsky, H. & Sinclair, J. (1984) Biochem. J. **222**, 737–748

Sinclair, P., Lambrecht, R. & Sinclair, J. (1987) Biochem. Biophys. Res. Commun. **146**, 1324–1329

Sinclair, P. R., Frezza, J., Sinclair, J., Bement, W., Haugen, S., Healey, J. & Bonkovsky, H. (1989) Biochem. J. **258**, 237–245

Smith, A. G. & Francis, J. E. (1983) Biochem. J. **214**, 909–913

Smith, A. G. & Francis, J. E. (1987) Biochem. J. **246**, 221–226

Sousa, R. & Marletta, M. (1985). Arch. Biochem. Biophys. **240**, 345–357

Sweeney, G. (1986) Clin. Biochem. **19**, 3–15

Sweeney, G. & Jones, K. G. (1977) Res. Commun. Chem. Path. Pharmacol. **17**, 631–637

Thomas, P. E., Korzeniowski, D., Ryan, D. & Levin, W. (1979) Arch. Biochem. Biophys. **192**, 524–532

Thomas, P. E., Reik, L. M., Ryan, D. E. & Levin, W. (1983) J. Biol. Chem. **288**, 4590–4598

Towbin, H., Staehelin, T. & Gordon, J. (1979) Proc. Natl. Acad. Sci. U.S.A. **76**, 4350–4354

Urquhart, A. J. & Elder, G. H. (1987) Biochem. Pharmacol. **36**, 3795–3796

Urquhart, A. J., Elder, G. H., Roberts, A. G., Lambrecht, R., Sinclair, P., Bement, W., Gorman, N. & Sinclair, J. (1988) Biochem. J. **253**, 357–362

Voorman, R. & Aust, S. (1987) Toxicol. Appl. Pharmacol. **90**, 69–78

Voorman, R. & Aust, S. (1988) Arch. Biochem. Biophys. **262**, 76–84

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